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EXHIBIT 5

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A NEW FILAMENTOUS PHAGE CLONING VECTOR: fd-tet

(Recombinant DNA; single-stranded DNA bacteriophages; tetracycline resistance transposon Tn*10*; restriction endonucleases; molecular cloning)

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SUMMARY

We have constructed a hybrid chromosome composed of the genome of wild-type fd (a filamentous, male-specific bacteriophage) and a segment of transposon Tn*10* coding for tetracycline resistance but not including the Tn*10* insertion sequences. The hybrid phage infects male *E. coli*, thereby transducing the infected cells to tetracycline resistance. The phage DNA can also be propagated in F⁻ cells after transfection. This new phage, fd-tet, may be used as a cloning vector to produce large quantities of cloned DNA in single-stranded form. Its usefulness has been demonstrated by cloning of a fragment from bacteriophage λ. Some unexpected sequence alterations have been identified in λ cloning experiments.

INTRODUCTION

For many experiments in molecular genetics it is necessary to prepare one of the strands of some DNA sequence of interest. Such isolated single strands can act as templates in DNA sequencing (Sanger et al., 1977) or site-specific mutagenesis (Hutchison et al., 1978), for example, or as strand-specific hybridization probes.

The filamentous Ff phages (for reviews see Ray, 1977; Denhardt, 1975) can serve as vectors for producing preparative quantities of cloned DNA in single-stranded form. The filamentous virion contains a single-stranded circular DNA genome; the length of the virion can vary to accommodate DNA strands of virtually any length. Heterologous DNA sequences can be inserted

Abbreviations: bp, base pairs; kbp, kilobase pairs; RF, replicative form; SDS, sodium dodecyl sulfate.

titered in the same way, except that the mixture is poured onto GBST plates, which contain tetracycline.

(c) Media

Mg-tet is M9 medium (Miller, 1972) supplemented with 50 mg/l of proline, leucine, threonine, tetracycline, and thiamine·HCl, and 20 g/l casamino acids. BFJ base is 4.5 g/l KH_2PO_4, 8.3 g/l Na_2HPO_4, 1 g/l NH_4Cl, 10 mg/l thiamine·HCl, 1 mM $MgSO_4$, and 0.03 mM $CaCl_2$. BBS is BFJ base supplemented with 12.6 g/l glycerol and 100 mg/l spectinomycin. GBS is BFJ base supplemented with 20 g/l glucose and 120 mg/l streptomycin; GBST is GBS supplemented with 5 mg/l tetracycline. BFJ-salts top-agar consists of the first three components of BFJ base supplemented with 7.5 g/l agar. NZY medium is 10 g/l NZ amine A (Humko-Sheffield), 5 g/l yeast extract, 5 g/l NaCl, and 2 g/l $MgCl_2 \cdot 6H_2O$; NZY-tet is NZY supplemented with 75 mg/l tetracycline. NZ top-agar is 5 g/l NaCl, 2 g/l $MgCl_2$, 10 g/l NZ amine A, and 7.5 g/l agar. YTMT medium is YT (Miller, 1972) supplemented with 20 mg/l of methionine and tetracycline. Plates were made by mixing equal volumes of autoclaved 44 g/l agar and a twofold concentrate of the medium and pouring about 20 ml into each 100 mm petri plate.

(d) DNA

Covalently closed circular pRT44 DNA was isolated after chloramphenicol amplification (Clewell, 1972) by a modified dye-buoyant-density method (Radloff et al., 1967).

Covalently closed circular RF form of fd and its derivatives were isolated as follows. Cells from 450 ml of an early stationary-phase culture were suspended in 50 ml of 25% (w/v) sucrose in 50 mM Tris, 5 mM EDTA, pH adjusted to 8.0 with HCl, in a 250-ml plastic bottle. Keeping the suspension cold, 13.3 ml of 0.4 M EDTA (pH adjusted to about 8 with NaOH) was added and the cells incubated for 20 min. Then 3.3 ml of 15 mg/ml hen egg-white lysozyme was added and the cells incubated a further 10 min. After adding 1.38 ml of 10% (w/w) Brij 58 and 0.56 ml of 5% (w/w) sodium deoxycholate, the suspension was incubated another 30 min, then frozen and thawed to complete lysis. The bottle was centrifuged at 14 000 × *g* for 2 h at 0°C to sediment the main chromosomal DNA. For each g of the clear supernatant, 0.098 ml of 5 mg/ml ethidium bromide in TES (10 mM Tris, 1 mM EDTA, 10 mM NaCl, pH adjusted to 8.0 with HCl) and 0.806 g solid CsCl were added. The solution was centrifuged for 48 h at 35 000 rev./min in the Beckman SW41 rotor at 20°C. The covalently closed circular fractions from several tubes were pooled and recentrifuged for 24—36 h at 40 000 rev./min in the Beckman SW50.1 rotor at 20°C. The ethidium bromide was removed from the covalently closed circular fraction by extraction with isopropanol, then the DNA was precipitated by adding 2 vol. TES, 1/3 vol. of 3 M sodium acetate (pH adjusted to 6 with acetic acid), and 6 vol. ethanol, and keeping the solution overnight at −20°C. The precipitate was wash-

(g) Splicing phage λ Hind*III fragments into fd-tet*

10 μg of a complete *Hin*dIII digest of fd-tet RF in 60 μl of *Hin*dIII buffer (20 mM Tris, 7 mM $MgCl_2$, 60 mM NaCl, and 0.2 μl/ml 2-mercaptoethanol, pH adjusted to 8.0 with HCl) was treated with 200 units of bacterial alkaline phosphatase (Bethesda Research Laboratories) at 65°C for 2 h. After a single phenol extraction according to Blin and Stafford (1976), the DNA was ethanol-precipitated and redissolved at 50 μg/ml in *Hin*dIII buffer. 8 μl of this solution (containing 400 ng of DNA) was mixed with 1.5 μg of a complete *Hin*dIII digest of phage λ DNA in 6 μl of *Hin*dIII buffer, 8 μl of 20 × ligase salts, and 48 μl of RI buffer. Two Weiss units of T4 DNA ligase were added and the ligation mixture incubated for 24 h in the refrigerator. This preparation was used to transfect K802 cells as described below.

(h) Transfection

DNA was diluted to 0.5 μg/ml in 0.1 M $CaCl_2$ and used to transfect K802 cells by the procedure of Cohen et al. (1972). Cells equivalent to 100 ng of DNA were then suspended in 1 ml of NZY medium supplemented with 0.5 μg/ml tetracycline and incubated at 37°C for 1 h to allow induction of the tetracycline-resistance gene before the transfection mixture was poured in about 3 ml of NZ top-agar onto an NZY-tet plate. Tetracycline-resistant colonies appeared 1—3 days later.

We did not plaque-purify the phages from transfected cells; rather, the transfected cells themselves were subcloned. Although this procedure does not directly clone the phage genome, it probably does so indirectly. Recipient cells, which are F^-, are not subject to reinfection. Thus, the entire population of phage genomes present in any given cell can only descend from the genomes present in the direct lineal ancestors of the original recipient. If we assume that the phage genomes segregating into each daughter at a cell division are a more or less random sample of the genomes in the mother cell, the small population of genomes in a single line of cells will undergo a sort of genetic drift that is exactly analogous to genetic drift in a small population of organisms. Because of this drift, variations rapidly disappear or become fixed in the population soon after they arise. The small population of genomes in one cell, therefore, ought to be heterogeneous only for recent variations that are still in the process of elimination or fixation.

RESULTS

Restriction maps of pRT44 (Jorgensen et al., 1979) and fd RF (Beck et al., 1978) are shown in Fig. 1. Both of these molecules are circular in the cell but are represented linearly here. pRT44 is shown as if it were cleaved at one of its two *Pst*I sites. The insertion sequences that flank the Tn*10* portion of pRT44 are cross-hatched in the map. Within the 2.8 kbp region that lies between the two *Bgl*II sites in Tn*10* there is a Tc gene, which confers tetracycline resistance to a host bacterium (Jorgensen et al., 1978). fd is shown as

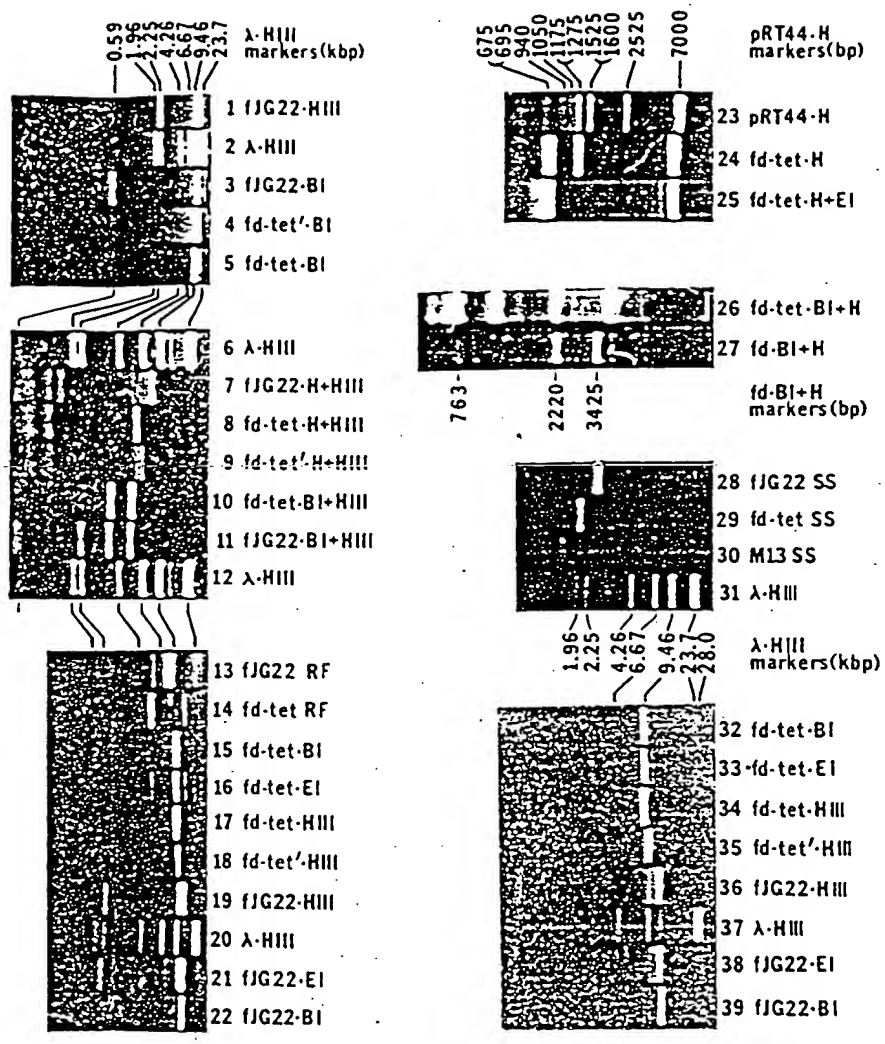

Fig. 2. Gel electrophoresis of viral strands, RFs, and restriction enzyme digests. The enzymes used in the digests are listed after the dots, the abbreviations being the same as in the legend to Fig. 1. SS stands for single viral strands. Agarose concentrations were 1.3% for lanes 1—5 and 23—25, 1% for lanes 13—22 and 26—31, and 0.7% for lanes 6—12 and 32—39.

37°C in a shaker-incubator. The supernatant of this culture had a titer of about $2 \cdot 10^{10}$ pfu and $4 \cdot 10^{10}$ tu per ml. Every plaque tested gave rise to a colony of tetracycline-resistant cells when transferred to a GBST plate, while cells in the lawn and in wild-type fd plaques failed to give rise to such colonies.

with *Hin*dIII and treated the digest with phosphatase to remove the 5′ phosphates. Such molecules should not be capable of recircularization by ligase, but should be able to be spliced to non-phosphatase-treated foreign DNA fragments (Ullrich et al., 1977). Accordingly, the phosphatase-treated digest of fd-tet RF was mixed with a *Hin*dIII digest of phage λ DNA, incubated with ligase, and used to transfect K802 cells. An average of 43 tetracycline-resistant colonies appeared per plate when these transfected cells were poured on NZY-tet plates; by contrast, if the phosphatase-treated *Hin*dIII digest of fd-tet was incubated with ligase in the absence of heterologous *Hin*dIII fragments, it gave rise to no tetracycline-resistant transfectant colonies. These results argue that most clones carry λ DNA inserts. We have made no attempt to increase the cloning efficiency; our conditions are probably far from optimal.

The single-stranded viral DNA was isolated from about 30 of the clones presumed to carry λ inserts and examined by gel electrophoresis. The viral strands from many of the clones migrated considerably more slowly than those of fd-tet, as expected if they carried any but the two smallest λ *Hin*dIII fragments. Electrophoresis of the viral strands from one of these clones, fJG22, is shown next to the viral strands of fd-tet and M13 (M13 is essentially identical to fd) in Fig. 2, lanes 28—30.

*Hin*dIII digestion of fJG22 RF released a fragment comigrating with the 2.25 kbp *Hin*dIII fragment of phage λ, as can be seen in Fig. 2, lanes 19 and 36. If it is indeed the 2.25 kbp λ *Hin*dIII fragment that is cloned in fJG22, then *Eco*RI digestion of fJG22 RF should also release a fragment of approx. 2 kbp, since there is an *Eco*RI site about in the middle of the 2.25 kbp λ *Hin*dIII fragment and another about 1 kbp away from the *Hin*dIII site in fd-tet; this prediction is confirmed in Fig. 2, lane 21. The 2.25 kbp fragment of λ has no *Bam*HI site, so fJG22 RF should have only a single *Bam*HI site (the one that is 3.8 kbp away from the *Hin*dIII site in fd-tet; see Fig. 3). In accord with this prediction, *Bam*HI cleavage of fJG22 RF does not release any fragments in the range of 3.8 to 7.6 kbp, as it would if there were a *Bam*HI site in the insert (Fig. 2, lane 22).

In all the foregoing respects, then, the restriction pattern of fJG22 strongly suggests that it carries the 2.25 kbp *Hin*dIII fragment of λ as an insert in its *Hin*dIII site. In other respects, however, the restriction pattern of fJG22 is anomalous. *Hin*dIII and *Eco*RI digestion of fJG22 should release a 9.2 kbp fragment that comigrates (approximately in the case of *Eco*RI) with *Hin*dIII-cleaved fd-tet; but in fact the major large fragment in the *Hin*dIII and *Eco*RI digests of fJG22 (Fig. 2, lanes 36 and 38, respectively) migrates close to the single large fragment in the *Bam*HI digest of fJG22 (lane 39), which — as expected — migrates as if it were about 2 kbp longer than fd tet. In both the *Hin*dIII and *Eco*RI digests of fJG22 (lanes 36 and 38), but not in the *Bam*HI digest (lane 39), there is a series of minor fragments migrating faster than the major approx. 11 kbp fragment. The fastest of these minor fragments migrates roughly coincidentally with *Hin*dIII-cleaved fd-tet.

Parallel anomalies are observed in the sizes of the largest fragments in a

We examined the restriction pattern of the fd-tet RF preparation (we call this preparation fd-tet′) that was used to clone λ fragments for evidence of reduplication of the 500 bp segment. A minor product of *Hin*dIII digestion of fd-tet′ RF comigrates with *Hin*dIII-cleaved fd-tet, but the major product migrates as if it were about 500 bp longer (Fig. 2, lane 35). The large minor and major *Hin*cII/*Hin*dIII fragments of fd-tet′ (lane 9) indicate the same thing. *Bam*HI digestion of fd-tet′ releases a small amount of a 500 bp fragment (lane 4). These lines of evidence suggest that the major fd-tet′ RF carries a duplication of the same 500 bp segment that is reduplicated up to 5 times in fJG22 RF.

As discussed below, the putative duplication in fJG22 must occur entirely within gene III, which is required for production of infectious phage. Accordingly, we spot-tested several subclones of K802(fJG22) for the presence of transducing particles by the same method used to test the original K802 (fd-tet) transfectants: some but not all subclones were positive for infective particles by this criterion.

DISCUSSION

Using the fd genome as a parent molecule, we have constructed a new phage, fd-tet, bearing a selectable marker: tetracycline resistance. There is a single *Hin*dIII site in the fd-tet RF, and *Hin*dIII fragments from heterologous DNA can be inserted into this site.

One preparation of fd-tet RF (the preparation we call fd-tet′) seems to carry a duplication of a short segment of fd-tet. When fd-tet′ was used to clone *Hin*dIII fragments from λ DNA, the RF from one of the transfectants, fJG22, seems to carry a quintuplication of the same segment. An examination of the nucleotide sequence of fd suggests a possible origin of this duplication. On either side of the *Bam*HI site, and situated about 500 bp apart, are two small tandem arrays of a 15 bp repeat coding for the amino acid sequence Glu-Gly-Gly-Gly-Ser (Schaller et al., 1978). Homologous but unequal crossover between these two arrays could generate the original duplication in fd-tet′. The quintuplication in fJG22 could easily have arisen from the duplication in fd-tet′ by a few additional rounds of homologous but unequal crossover.

Whatever its origin, the duplicated segment in fJG22 and fd-tet′ is no more than 590 bp long and must include the *Bam*HI site in gene III. Since that *Bam*HI site is at least 620 bp away from both ends of gene III, the duplicated segment presumably lies entirely within gene III. It is likely, therefore, that the genomes bearing this duplication have an inactive gene III.

Pratt et al. (1969) characterized chain-terminating mutants in gene III in the closely related phage M13. They found that nonsuppressing male cells infected with any gene III amber mutant were killed; cells infected with nonpolar gene III amber mutants secreted noninfectious phages before cell death,

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